BEST AVAILABLE COPY

PCT/GB2004/000690

REC'D 10 MAY 2004
WIPO PCT

PA 1150536

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

April 07, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: ***60/462,269***
FILING DATE: ***April 11, 2003***

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. SWAIN
Certifying Officer

04-14-03 60462269 .041103 # 4/pro

Page 43 i.e abstract is Under Claim

JC602 U.S. PTO 04/11/03

PATENT

PROVISIONAL APPLICATION FOR PATENT COVER SHEET
(37 CFR §1.51(c)(1))

JC879 U.S. PTO 60/462269 04/11/03

Box: Provisional Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Title of Invention: Methods and Means for Treating Protein Conformational Disorders

Inventors:

Full Name	Citizenship	Address
David Rubensztein	Great Britain	Cambridge Institute for Medical Research Wellcome/MRC Building Addenbrookes Hospital, Hills Rd Cambridge CB2 2XY UK
Brinda Ravikumar	India	Cambridge Institute for Medical Research Wellcome/MRC Building Addenbrookes Hospital, Hills Rd Cambridge CB2 2XY UK
Julie Webb	Great Britain	Cambridge Institute for Medical Research Wellcome/MRC Building Addenbrookes Hospital, Hills Rd Cambridge CB2 2XY UK

Correspondence Address: Please address all correspondence to the attorney named below.

Attorney Docket No.: 40225001/NRS/CP6141964

Enclosed Application Parts:

- Application Text -- 45 pages total (including drawings, if any)
- Form PTO-2038 authorizing charge for provisional filing fee (37 CFR §1.16(k)): $160

Government Rights: The invention was made by, or under a contract with, the following agency of the United States Government: NONE.

The Commissioner is authorized to charge any fees or credit any overpayments to Deposit Account Number 18-2055.

For the Applicant,

Craig A. Fieschko, Reg. No. 39,668
DEWITT ROSS & STEVENS, S.C.
Firstar Financial Centre
8000 Excelsior Drive, Suite 401
Madison, Wisconsin 53717-1914
Telephone: (608) 831-2100
Facsimile: (608) 831-2106

I certify that this correspondence is being deposited with the United States Postal Service as Express Mail - Post Office to Addressee, in an envelope addressed to: Box: Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

EL901768933US	4-11-03	Marcia Layton
Express Mail Label No.	Date of Deposit	Signature

GB 0462269 .031103

Methods and Means for Treating Protein Conformational Disorders

This invention relates to compositions and methods for the treatment of a range of conditions that are characterised by the formation of intracellular protein aggregates (known as Protein Conformational Disorders), including Huntington's disease.

Proteinopathies or Protein Conformational Disorders (PCDs) are associated with particular proteins or sets of proteins that misfold and aggregate in specific tissues (Paulson,H.L. (1999) Am. J. Hum. Genet. 64, 339-345). Some PCDs are caused by codon reiteration mutations, where protein misfolding is mediated by the abnormal expansion of a tract of repeated amino acids. These include polyglutamine (polyQ) expansion diseases, exemplified by Huntington's disease (HD). HD is characterised by expansions of a polyQ stretch in exon 1 of the Huntington gene to more than 37 glutamines, and a short N-terminal fragment encoding the polyglutamine stretch is sufficient to cause aggregates in mice (Schilling,G. *et al.* (1999) Hum. Mol. Genet., 8, 397-407) and in cell models (Wyttenbach,A. et al (2000) Proc. Natl. Acad. Sci. U S A, 97, 2898-2903). It is commonly believed that the mutant protein acquires its toxicity and its propensity to aggregate after cleavage, forming a short (so far, incompletely-defined) N-terminal fragment containing the polyglutamine stretch (Martindale,D. *et al.*, (1998) Nat. Genet., 18, 150-154). More recently, polyalanine (polyA) expansion mutations in the polyadenine-binding protein 2 gene have been shown to cause OPMD, which is associated with aggregates in muscle

cell nuclei (Brais, B *et al.*(1998). Nat. Genet.18, 164-167). This disease has been modelled in cell culture systems where aggregate formation is associated with cell death (Fan,X et al (2001) Hum. Mol. Genet., 10, 2341-2351). PolyA expansions of 19 or more repeats tagged with enhanced green fluorescent protein are sufficient to cause intracytoplasmic aggregate formation and cell death in cultured cells (Rankin,J. et al (2000) Biochem. J., 348, 15-19). Many of the codon reiteration diseases are dominantly inherited and genetic and transgenic studies suggest that they are generally due to gain-of-function mutations (for instance in polyQ diseases) (Narain,Y. et al (1999). J. Med. Genet., 36, 739-746).

Parkinsons disease (PD) is caused by the degeneration of dopaminergic neurons in the substantia nigra. The pathogenic hallmark of PD is the accumulation and aggregation of α-synuclein in susceptible neurons. The cytoplasmic aggregates/inclusions characteristic of PD are called Lewy bodies and their major constituent is α-synuclein (Kahle, P.J. et al (2002) *J. Neurochem.* **82**, 449457). Lewy pathology is also found in dementia with Lewy Bodies (LB), the LB variant of Alzheimer's disease, in neurodegeneration with brain iron accumulation type I and in glial cytoplasmic inclusions of multiple system atrophy. These diseases are collectively known as α-Synucleinopathies (Spillantini, M. G. et al (1997) *Nature* **388**, 839840, Mezey, E et al . (1998) *Mol. Psychiatry* **3**, 493499).

In many cases, disease severity correlates with the expression levels of the mutant protein. Factors regulating clearance of these aggregate-prone proteins and of the aggregates themselves may therefore play an

important role in the disease. Aggregate-prone proteins are generally believed to be cleared by the ubiquitin-proteasome pathway, where malfolded proteins are tagged with ubiquitin, which serves to route them to the proteasome for degradation. The role of this pathway in polyQ diseases is supported by the observations that these proteins are ubiquitinated and that the aggregates are associated with proteasomal subunits (Wyttenbach et al (2000) supra; Jana, N.R. et al (2001) Hum. Mol. Genet. 10, 1049-59; Suhr S.T. et al (2001) J. Cell Biol. 153, 283-294). Furthermore, inhibition of the proteasome with lactacystin, or its more potent derivative beta-lactone, resulted in increased inclusion formation (Wyttenbach et al (2000) supra; Waelter,S. et al (2001) Mol. Biol. Cell, 12, 1393-1407; Bence,N.F. et al (2001) Science, 292, 1552-1555.).

SCANNED, #____

The lysosomes, which are often considered as non-specific systems for protein degradation, have recently been shown to be able to selectively receive and degrade certain intracellular proteins. The process of bulk degradation of cytoplasmic proteins or organelles in the lytic compartment is termed autophagy. It involves the formation of double membrane structures called autophagosomes or autophagic vacuoles, which fuse with the primary lysosomes where their contents are degraded and then either disposed off or recycled back to the cell (Klionsky,D.J. and Oshumi,Y. (1999) Annu. Rev. Cell Dev. Biol. 15, 1-32).

The present inventors have recognised that the autophagy-lysosome pathway is a major route for the degradation of aggregate-prone proteins and aggregates. Inhibition of autophagy increased the levels and rate of aggregate

formation, while increased aggregate clearance was associated with the stimulation of autophagy by rapamycin. This finding has significant application in the treatment of Protein Conformational Disorders, in which the formation of aggregates is closely associated with toxicity.

Various aspects of the invention relate to methods and means for the treatment of protein conformational disorders, including codon reiteration mutation disorders and α-synucleinopathies, by stimulation of autophagic activity.

An aspect of the invention provides a method of treating a protein conformational disorder in an individual comprising: stimulating autophagy activity in the cells of the individual.

Protein conformational disorders are characterised by the intracellular accumulation of protein aggregates. Aggregates may accumulate, for example, in the cytoplasm of a cell. Commonly, aggregates may form in neuronal cells, such as brain cells, for example in disorders such as Huntington's disease and Parkinson's disease.

Protein conformational disorders which may be treated in accordance with the invention include codon reiteration mutation disorders, in particular polyQ expansion disorders such as Huntington's disease, spinocerebellar ataxias types 1, 2, 3, 6, 7, and 17, Kennedy's disease and dentatorubral-pallidoluysian atrophy. These disorders are characterised by the aggregation of mutant proteins which contain an expanded tract of repeated glutamine

residues. For example, HD is characterised by an expanded polyQ stretch in exon 1 of the Huntington gene.

Protein conformational disorders which may be treated in accordance with the invention also include polyA expansion disorders. These disorders are characterised by the aggregation of mutant proteins which contain an expanded tract of repeated alanine residues. For example, oculapharyngeal muscular dystrophy (OPMD) is characterised by a polyadenine (polyA) expansion mutation in the polyadenine binding protein 2 gene.

Other protein conformational disorders which may be treated in accordance with the invention may include α-synucleiopathies such as Parkinson's disease, LB variant Alheimer's disease and LB dementia. These are disorders characterised by the accumulation of cytoplasmic aggregates called Lewy bodies, which comprise α-synuclein.

Protein conformational disorders that may be treated in accordance with the invention also include prion disorders such as CJD.

The enhancement of the autophagic/lysomal pathway is shown herein to mediate the clearance of protein aggregates, in particular cytoplasmic protein aggregates, which are associated with toxicity in protein conformational disorders.

Various methods of stimulating the activity of the autophagic/lysomal pathway are possible. For example, an individual may be subjected to a leucine-free dietary

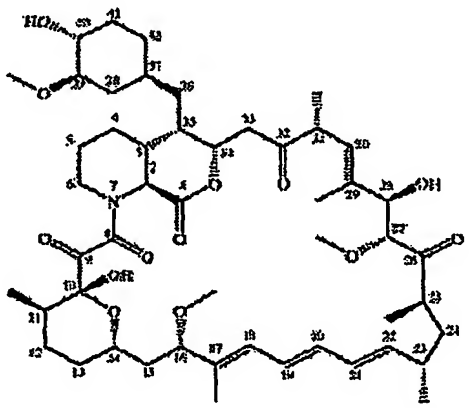
regime or an agent or composition which stimulates autophagy may be administered.

In preferred embodiments, activity may be stimulated or enhanced in the cells of the individual by administering an autophagy-inducing agent to said individual.

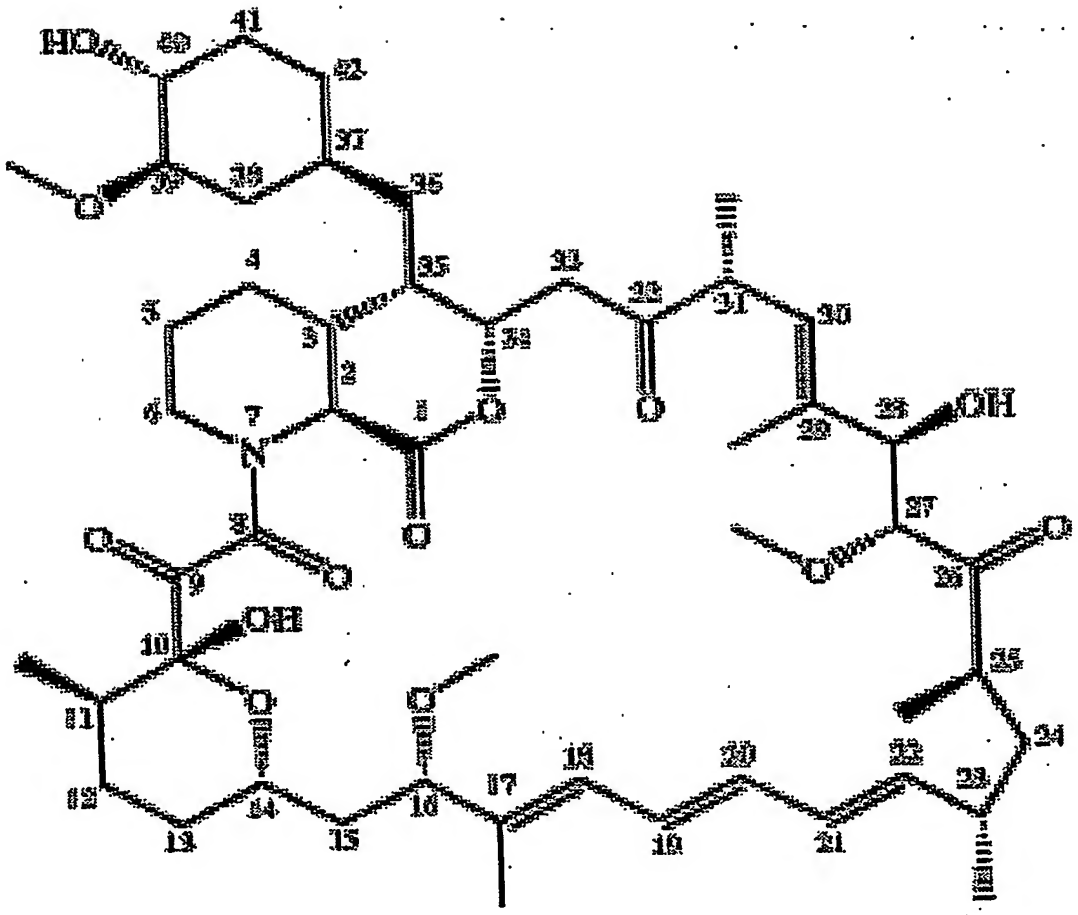
An autophage inducing agent may be any compound or molecule which stimulates or induces the activity of the autophagic/lysomal pathway within cells. Particularly suitable autophagy inducing agents include rapamycin macrolides such as rapamycin and its numerous analogues and derivatives.

Rapamycin and its derivatives and analogues are lactam macrolides. A macrolide is a macrocyclic lactone, for example a compound having a 12-membered or larger lactone ring. Lactam macrolides are macrocyclic compounds which have a lactam (amide) bond in the macrocycle in addition to a lactone (ester) bond.

Rapamycin is produced by Streptomyces hygroscopicus, and has the structure shown below.



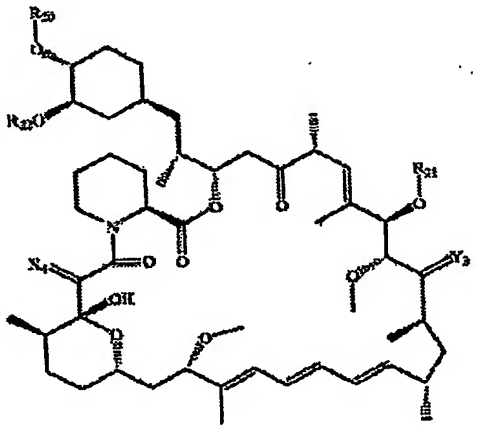
(A)



[Enlargement of diagram from prior page]

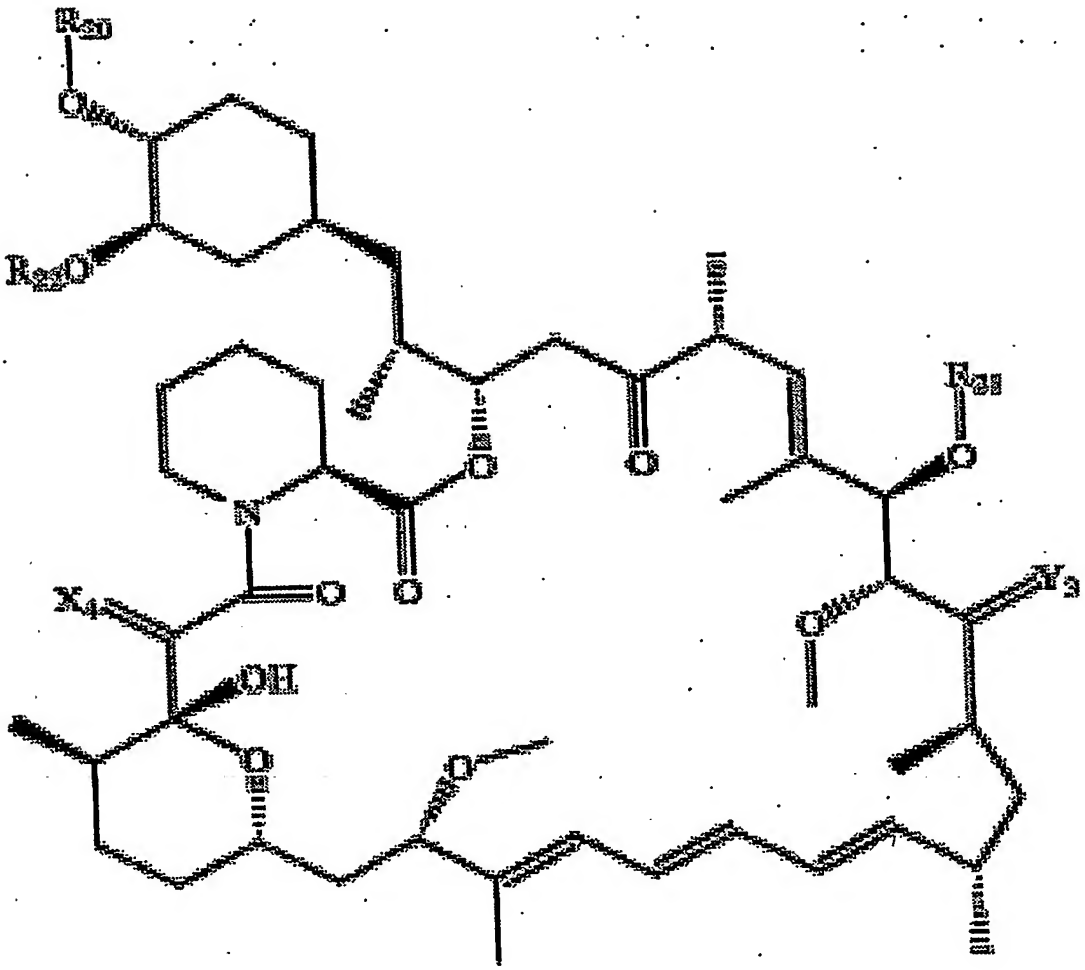
See, e.g., McAlpine J.B. *et al.* J.Antibiotics (1991) 44: 688; Schreiber,S.L. *et al.* J. Am. Chem. Soc.(1991) 113:7433; US3,929,992.

One group of rapamycin analogues are 40-0-substituted derivatives of rapamycin having the structure set out below;



wherein; X_4 is (H,H) or O; Y_3 is (H,OH) or O; R_{20} and R_{21} are independently selected from H, alkyl, arylalkyl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkoxycarbonylalkyl, hydroxyalkylaryalkyl, dihydroxyalkylarylalkyl, acyloxyalkyl, aminoalkyl, alkylaminoalkyl, alkoxycarbonylaminoalkyl, acylaminoalkyl, arylsulfonamidoalkyl, allyl, dihydroxyalkylallyl, dioxolanylallyl, dialkyl-dioxolanylalkyl, di(alkoxycarbonyl)-triazolyl-alkyl and hydroxyalkoxy-alkyl;

wherein "alk-" or "alkyl" refers to C_{1-6} alkyl, branched or linear, preferably C_{1-3} alkyl,; "aryl" is phenyl or tolyl; and acyl is a radical derived from a, carboxylic acid; and;



[Enlargement of diagram from prior page]

R_{22} is methyl or R_{22} and R_{20} together form C_{2-6} alkyl; provided that R_{20} and R_{21} are not both H; and hydroxyalkoxyalkyl is other than hydroxyalkoxymethyl.

Suitable rapamycin analogues are disclosed in WO 94/09010 and WO 96/41807.

Particularly suitable rapamycin analogues include 40-O-(2-hydroxy)ethyl-rapamycin, 32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-40-O-(2-hydroxyethyl)-rapamycin, 16-O-pent-2-ynyl-32-(S)-dihydro-rapamycin and 16-O-pent-2-ynyl-32-(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin.

Other rapamycin analogues include carboxylic acid esters as set out in WO 92/05179, amide esters as set out in US5,118,677, carbamates as set out in US5,118,678, fluorinated esters as set out in US5,100,883, acetals as set out in US5,151,413, silyl ethers as set out in US5,120,842 and arylsulfonates and sulfamates as set out in US5,177,203.

Other rapamycin analogues which may be used in accordance with the invention may have the methoxy group at the position 16 replaced with alkynyloxy as set out in WO 95/16691. Rapamycin analogues are also disclosed in WO 93/11130, WO 94/02136, WO 94/02385 and WO 95/14023.

Rapamycin and its analogues are known to possess immunsuppressive activity and are in clinical use for the treatment of transplant rejection (Chan, L. et al (2001) Am. J. Kidney Dis., 38, S2-S9).

Another aspect of the invention provides the use of an

autophagy inducing agent in the manufacture of a medicament for use in the treatment of a protein conformational disorder.

Protein conformational disorders and autophagy inducing agents are described in detail above.

Another aspect of the invention provides a pharmaceutical composition for use in the treatment of a protein conformational disorder comprising a rapamycin macrolide and a pharmaceutically acceptable excipient, vehicle or carrier.

A pharmaceutically acceptable excipient, vehicle or carrier, should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles

such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Examples of techniques and protocols mentioned above can be found in Remingtonﾒs Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

Formulations and administration regimes which are suitable for use with rapamycin macrolides such as rapamycin are well known in the art.

Administration is preferably in a "therapeutically effective amount", this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of medical practitioners.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated

Other aspects of the invention provide screening methods for agents useful in treating protein conformational disorders.

A method of identifying an agent useful in the treatment of a protein conformational disorder may comprise;

contacting a mammalian cell with a test compound; and,

determining the autophagy activity of said cell, an increase in autophagy activity in the presence of said compound being indicative that the compound is a

candidate agent for use in the treatment of a protein conformational disorder.

A method may include determining the ability of said compound to increase the clearance of cytoplasmic protein aggregates. This may be determined for example, in the presence of proteosome inhibitors such as epoximicin.

Any suitable mammalian cell may be used, for example Chinese hamster ovary cells, baby hamster kidney cells, COS cells, PC12 and many others.

Autophagy activity may be determined by any convenient method, for example monodansylcadaverine (MDC) staining (Ravikumar et al Hu. Mol. Gen. (2003) 12 9 1-10), LC3 processing (Y. Kabeya et al EMBO J. 19 5720-5728), or electron microscopy visulating autophagosome numbers.

Test compounds may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

Combinatorial library technology (Schultz, JS (1996) Biotechnol. Prog. 12:729-743) provides an efficient way of testing a potentially vast number of different substances for ability to modulate activity of a polypeptide. Suitable test compounds may be based on rapamycin i.e. rapamycin derivatives or analogues.

The amount of test substance or compound which may be added to an assay will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.1 to 100 μM concentrations of

putative inhibitor compound may be used, for example from 1 to 10 μM. the test substance or compound is desirably membrane permeable in order to access the intracellular components of the autophagy/lysosome pathway.

Following identification of a compound as described above, a method may further comprise modifying the compound to optimise the pharmaceutical properties thereof.

Such a method may comprise determining the autophagy inducing activity of the modified compound.

For example, a method of producing an agent for the treatment of a protein comformational disorder may comprise;
modifying rapamycin to produce a derivative; and,
determining the autophagy inducing activity of said derivative.

The modification of a 'lead' compound identified as biologically active is a known approach to the development of pharmaceuticals. Modification of a known active compound (such as rapamycin) may be used to avoid randomly screening large number of molecules for a target property.

Modification of a 'lead' compound to optimise its pharmaceutical properties commonly comprises several steps. Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR.

Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the modified compound is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the autophagy inducing activity of the lead compound. The modified compounds found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Modified compounds include mimetics of the lead compound.

Further optimisation or modification can then be carried out to arrive at one or more final compounds for *in vivo* or clinical testing.

The test compound may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals, e.g. for any of the purposes discussed elsewhere herein.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. All documents mentioned in this specification are incorporated herein by reference in their entirety.

Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures described below.

Figure 1 shows the increases in aggregation and cell death caused by 3-methyl adenine (3-MA) in transient transfections COS-7 cells expressing an exon 1 fragment of the HD gene with 74 glutamines (Q74) or enhanced green fluorescent protein (EGFP) fused to 19 alanines (A19). The percentages of Q74- or A19-transfected COS-7 cells with aggregates and abnormal nuclei are shown 48 hours after transfection with Q74 (top) or A19 (bottom) without (-) or with (+) treatment with 10mM 3-MA for 15 hours prior to fixing.

Figure 2 shows that Bafilomycin A1 (BafA1) increases aggregation and cell death in COS-7 cells expressing Q74 or A19. The percentages of EGFP-positive cells with Q74 or A19 aggregates and abnormal nuclear morphology are shown after 48 hours of transfection with (+) or without (-) 200nM BafA1. BafA1 was added 15h before fixing cells. CON = control, BafA1 = Bafilomycin A1.

Figure 3 shows that Rapamycin reduces aggregation and cell death in COS-7 cells expressing Q74 or A19. Figure 3A shows the percentages of Q74 or A19 transfected COS-7 cells after 48 hours with aggregates and abnormal nuclear morphology. CON = control; RAP = rapamycin (at 0.2µg/ml

final concentration added 15 hours prior to fixing). Figure 3B shows the percentages of EGFP-positive COS-7 cells with Q74 aggregates and cell death after 24 hours of transfection. Here rapamycin was added as in A.

Figure 4 shows that the effect of epoxomicin on COS-7 cells transfected with Q74 or A19. The percent of Q74- or A19-transfected cells with aggregates and nuclear abnormalities is shown with and without 10μM epoxomicin added 15 hours before fixing the cells. CON = control, Epox = epoxomicin.

Experimental

Materials and Methods

Plasmids

Mammalian expression vectors comprising EGFP (pEGFP-C1, Clontech) fused at its C-terminus with a Huntington's Disease gene exon 1 fragment with 74 polyglutamine repeats (Q74) or a polyalanine stretch of 19 repeats (A19) were used (Rankin,J. et al (2000) Biochem. J., 348, 15-19; Narain,Y. et al (1999) J. Med. Genet., 36, 739-746.). A Haemagglutinin-tagged Huntington's Disease gene exon 1 fragment with 74 polyglutamine repeats in pHM6 vector (Q74-HA) was also used. The PC12 stable lines expressing exon 1 of Huntington gene were as described in Wyttenbach A. *et al.*, (2001) Hum. Mol. Genet., 10, 1829-45.

Mammalian cell culture and transfection

African green monkey kidney cells (COS-7) were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin/Streptomycin, 2mM L-Glutamine and 1mM Sodium

Pyruvate at 37°C, 5% Carbon dioxide. The cells were grown on coverslips in six-well plates for immunofluorescence analysis, or were directly grown in six-well plates to 60-80% confluency for 24 hours for western blot analysis. Transfection was done using LipofectAMINE reagent (Invitrogen) using the manufacturer's protocol. The transfection mixture was replaced by normal culture medium after 5 hours incubation at 37°C and the transfected cells were analysed by immunofluorescence or immunoblot 48 hours after transfection. The cells were left untreated or treated with 10mM 3-methyl adenine (3-MA, Sigma), 0.5mM N^6, N^6, Dimethyl adenosine (DMA, Sigma), 0.2µg/ml Rapamycin (Sigma), 200nM Bafilomycin A1 (BafA1, Sigma), 10µM Lactacystin (Sigma) or 10µM epoxomicin (Affinity research products ltd.) for 15 hours before fixation for immunofluorescence or processing for western blots. DMA, BafA1, epoxomicin and rapamycin were dissolved in DMSO and 3-MA and lactacystin were in water. Equal amounts of water or DMSO were added to the untreated controls, where relevant. The cells on coverslips were rinsed with 1×PBS, fixed with 4% paraformaldehyde in 1×PBS for 20 min and mounted in antifadent supplemented with 4', 6-diamidino-2-phenylindole (DAPI, 3µg/ml, Sigma) to allow visualisation of nuclear morphology. The PC12 stable cells were maintained at 75 µg/ml hygromycin in standard medium consisting of high glucose DMEM (Sigma) with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine (Invitrogen), 10% heat-inactivated horse serum (Invitrogen), 5% Tet-approved fetal bovine serum (FBS) (Clontech) and 100µg/ml G418 (Invitrogen) at 37°C, 10% CO2. The cells were seeded at 1-2×10^5 per well in 6-well plates and were induced with 1µg/ml doxycycline (Sigma) for 8 hours. The expression of transgenes was switched off by removing doxycycline from

the medium. Cells were either left untreated or treated with 3-MA, BafA1, epoxomicin, lactacystin, rapamycin, 10μg/ml cycloheximide (Sigma) or cycloheximide + rapamycin at the concentrations specified above for 24, 48 or 72 hours and the medium with the inhibitors/activator changed every 24 hours. The cells were then scraped off from the wells into 1.5ml eppendorf tubes, pelleted at 8000 rpm and washed twice with 1×PBS. They were either fixed with 4% paraformaldehyde for 20 minutes, mounted in DAPI over coverslips on glass slides or processed for western blot analysis.

Western Blot Analysis of PolyQ and PolyA expression products

The pellets for westerns from COS-7 or PC12 cells were lysed on ice in Laemmli buffer (62.5mM Tris-HCl pH6.8, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) for 30min in the presence of protease inhibitors (Roche Diagnostics). The lysates were subjected to SDS-PAGE (10%) electrophoresis and proteins transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech). The primary antibodies used include mouse monoclonal anti-GFP antibody (8362-1, Clontech) at 1:2000, rabbit monoclonal anti-actin antibody (A2066, Sigma) at 1:3000, mouse monoclonal anti-tubulin antibody (Clone DM 1A, Sigma) at 1:1000 and mouse monoclonal anti-HA antibody (Covance) at 1:1000. Blots were probed with Horse Radish Peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG (Bio Rad) at 1:2000. Bands were visualised using the ECL detection reagent (Amersham).

Quantification of aggregate formation and abnormal cell nuclei

Aggregate formation and nuclear morphology were assessed using a fluorescence microscope. 200 EGFP positive COS-7 cells were selected and the proportion of cells with aggregates was counted. Aggregates are described in Rankin et al (2000) and Narain et al (1999) supra. Cells were considered dead if the DAPI-stained nuclei showed apoptotic morphology (fragmentation or pyknosis). Pyknotic nuclei are typically <50% diameter of normal nuclei and show obvious increased DAPI intensity. These criteria are specific for cell death, as they show a very high correlation with propidium iodide staining in live cells). Furthermore, these nuclear abnormalities are reversed with caspase inhibitors (Wyttenbach,A.et al (2000) Proc. Natl. Acad. Sci. U S A, 97, 2898-2903). Analysis was performed with the observer blinded to the identity of the slides and all experiments reported in the figures were done in triplicate at least twice.

Statistical Analysis

The P-values were determined by unconditional logistical regression analysis using the general loglinear analysis option of SPSS Ver. 6.1. software (SPSS, Chicago, USA).

Establishment of inducible α-synuclein expressing PC12 cell lines

Human α-synuclein with a HA tag at the N-terminus and a 6His tag at the C-terminus was amplified from pHM6α-synuclein constructs (Furlong, R. A. et a;l (2000) *Biochem. J.* 346, 577581) and inserted into pTRE2hyg vector (Clontech) using Nhe I/Sal I sites. Constructs were confirmed by sequencing before use. The

pTRE2hygα-synuclein construct and pTettTS (Clontech) were cotransfected into PC12 TetOn cells (Clontech), using Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen). Single colonies were isolated using cloning cylinders (Sigma) and cells were grown and tested for α-synuclein expression upon induction. Cell lines were subjected to a further round of purification from single colonies to ensure that lines were pure.

Cell culture

PC12 cells were grown in Dulbecco's modified eagle's medium (DMEM, Sigma) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), 100U/ml penicillin/streptomycin, 2mM L-glutamine, 50mg/ml G418 (Invitrogen) and 149mg/ml hygromycin B (Calbiochem) at 37°C, 10% CO_2. To induce differentiation, cells were grown in media containing 1% horse serum and 100ng/ml nerve growth factor (2.5S, Upstate Biotechnology) and incubated for about 5 days. Cells were induced to express synuclein with 2mg/ml doxycycline (Sigma).

Immunofluorescence

Coverslips were placed in 6 well dishes and coated with 0.01% poly-L-lysine (Sigma). Cells were seeded and induced with 2mg/ml doxycycline and incubated as necessary. Cells were fixed with 4% paraformaldehyde (Sigma) for 30 minutes, then washed with PBS and permeabilised with 0.1% Triton X100 (Sigma) for 15 minutes. Cells were blocked in 10% fetal calf serum for at least 30 minutes. Anti α-synuclein monoclonal antibody (BD Biosciences) was used at 1:200 for 2-16 hours, cells were washed and 1:200 Cy3conjugated antimouse antibody (Jackson ImmunoResearch labs) was added for 1 hour. This anti α-synuclein antibody could detect both

human and rat α-synuclein, therefore uninduced controls were performed in parallel to compare endogenous α-synuclein levels. Staining was always considerably fainter in the uninduced controls. For double staining with LysoTracker Red (Molecular Probes) cells were incubated in Earle's Balanced Salts Solution (Sigma) with 75nM LysoTracker for 2 hours at 37°C. Cells were fixed and stained with anti α-synuclein antibody and 1:200 FITCconjugated antimouse antibody (Jackson ImmunoResearch labs) was used as secondary antibody. Slides were mounted in Citifluor (Citifluor Ltd) with 3µg/ml 4',6' Diamidino-2-phenylindole (DAPI, Sigma). Cells were visualised using a Zeiss LSM510 confocal microscope.

Treatment with autophagy/proteasome drugs

Cells were induced for 24 hours and then washed twice with media to remove doxycycline. Then cells were incubated in media containing either 10mM 3-methyladenine (3MA, Sigma), 200nM bafilomycin A1 (Sigma), 0.2mg/ml rapamycin (Sigma), 10mM lactacystin (Sigma), 10 mM epoxomicin (Affinity Research Products Ltd) or carrier controls (water or DMSO (Sigma)). 3MA and lactacystin were made up in water and rapamycin, epoxomicin and bafilomycin were dissolved in DMSO.

After 24 hours the media was replaced with fresh media plus drug and after a further 24 hours cell pellets were collected and stored at -80°C until required.

Western blot analysis of α-Synuclein Expression

Cell pellets were collected and stored at -80°C until needed. Cells were lysed on ice in lysis buffer: 1% TritonX100, 20mM TRIS pH7.5, 137mM NaCl, 1mM EGTA,

10% glycerol, 1.5mM MgCl2 and protease inhibitor cocktail (Complete, Roche).

Samples were then mixed with loading buffer: 62.5mM TRIS pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 100mM dithiothreitol and 700mM β-mercaptoethanol and boiled before loading onto 14% denaturing polyacrylamide gels.

Each lane was loaded with protein from a similar number of cells, based on cell counting at the time of seeding. Proteins were transferred onto Hybond ECL nitrocellulose membrane (Amersham). α-Synuclein was detected with an antiHA monoclonal antibody (Covance) at 1:1000 dilution with 4-18 hours incubation. HRP conjugated antimouse antibody (Amersham) at 1:2000 dilution was then added to blots and antibody was detected using ECL blotting reagents (Amersham) and Hyperfilm ECL (Amersham). Blots were stripped and then reprobed for α-tubulin (Sigma) at 1:1000 dilution.

Immunogold labelling for electron microscopy

Cells were induced with 2mg/ml doxycycline for 48 hours. The cells were fixed *in situ* with 2% formaldehyde and 0.05% glutaraldehyde in 0.1M PIPES buffer and harvested by scraping. The cells were incubated in 0.1M PIPES buffer containing 5% BSA and 20% polypropylene glycol, concentrated by centrifugation at 2000rpm in a Hermle Z 160 M centrifuge (Hermle Labortechnik) and the supernatant was removed.

Small droplets (5 ml) of the cells were mounted onto aluminium foil and quench frozen, by plunging them into melting propane cooled in liquid nitrogen. After

freezing, the cells were transferred into a Leica AFS freeze substitution unit in vials of frozen, dry methanol, containing 0.5% uranyl acetate. They were maintained at -90°C for 24 hours followed by 24 hours at -70°C and another 24 hours at -50°C. They were infiltrated with Lowicryl HM20 over 3 days and polymerised by irradiation with UV light for 48 hours. Thin sections were cut using a Leica Ultracut S and mounted on Formvar coated nickel grids. The sections were incubated overnight in mouse AntiHA primary antibodies (Covance), diluted 1:5 in Tris Buffered Saline (TBS) at pH 7.4 containing 0.1% Tween 20, 0.1% Triton X100, 0.5% fetal calf serum and 10% normal goat serum. The sections were washed six times in TBS and incubated with goat antimouse immunoglobulins conjugated to 10nm gold particles (British Biocell), diluted 1:100 in the diluent for the primary antibody at pH 8.5 without added goat serum for 1 hour (25). They were rinsed 6 times in TBS and twice in deionised water and stained with uranyl acetate and lead citrate before viewing in a Philips CM100 transmission electron microscope.

Results

Inhibition of the sequestration stage of autophagy increases aggregate formation and cell death in COS-7 cells expressing mutated HD exon 1 or polyalanine protein

An exon 1 fragment of the Huntington's disease (HD) gene with 74 or 55 glutamines (Q74/Q55) fused to enhanced green fluorescent protein (EGFP) was analysed. The effects of perturbing autophagy on the HD constructs were compared with another aggregate-prone protein - EGFP fused to 19 alanine repeats (A19). Both the Q74/55 and A19 constructs form aggregates and are associated with

increased cell death, compared to Q23 and A7 constructs or empty EGFP vectors, which do not form any aggregates under the same expression conditions (Wyttenbach,A. et al (2000) Proc. Natl. Acad. Sci. U S A, 97, 2898-2903; Rankin,J. et al (2000) Biochem. J. 348, 15-19).

The role of autophagy in degrading these proteins was examined using the specific inhibitor 3-methyl adenine (3-MA) (Kovács,A.L et al (1998) Biol. Chem. 379, 1341-1347). 3-MA inhibits autophagy at the sequestration stage, where a double membrane structure forms around a portion of the cytosol and sequesters it from the rest of cytoplasm to form the autophagosome (Klionsky,D.J. and Oshumi,Y. (1999) Annu. Rev. Cell Dev. Biol. 15 1-32). 3-MA treatment resulted in an obvious change in the appearance of the aggregates formed by Q74 or A19 constructs transiently transfected into COS-7 cells. In cells with aggregates, 3-MA increased their apparent size and number. 3-MA treatment also increased the proportions of Q74- or A19-expressing cells with aggregates and this was accompanied by an increase in cell death (Fig. 1). 3-MA did not cause aggregate formation in COS-7 cells expressing EGFP tagged wild-type HD exon 1 protein with 23 glutamine repeats or an EGFP-polyalanine protein with 7 repeats. Identical aggregate phenotypes and similar significant increases in the proportions of Q74- and A19-transfected cells with aggregates or cell death were observed after treatment with another inhibitor of the sequestration stage of autophagy, N^6, N^6-dimethyladenosine (DMA). Similar results were obtained with Q55 and Q74). The increased aggregation of Q74 and A19 caused by 3-MA was associated with an increase in the levels of the transgene protein (about 80% transfection efficiency was obtained in COS-7 cells). 3-MA treatment

did not cause any change in the levels of empty EGFP transfected in the same way into COS-7 cells. However it did cause an increase in the levels of an HA-tagged version of the same HD exon 1 fragment with Q74. Thus, the 3-MA is not acting simply on the EGFP part of the fusion proteins we have studied. These results provide indication that inhibiting the sequestration stage of autophagy results in increased levels of the mutant proteins, thereby enhancing their aggregation.

Inhibition of autophagosome-lysosome fusion increases aggregation of Q74 or A19 in COS-7 cells

After the sequestration step, the autophagosome needs to fuse with the lysosome in order for its contents to be degraded. This next step was tested with the vacuolar ATPase inhibitor Bafilomycin A1 (BafA1), which interferes with the autophagosome-lysosome fusion step, possibly because lysosomal acidification is required for this fusion (Yamamoto, A. et al (1998) Cell Struct. Funct. 23, 33-42). As seen in Fig. 2, treatment with BafA1 resulted in a change in aggregate morphology (increased size) and increased the proportions of transfected Q74 or A19 COS-7 cells with aggregates similar to the results with 3-MA. This was also accompanied by an increase in cell death in these cells.

3-MA and BafA1 decrease turnover of Q74 aggregates in stable inducible PC12 cells

A stable doxycycline-inducible PC12 cell line expressing EGFP-tagged HD exon 1 with Q74 (Wyttenbach,A. *et al.*, (2001) Hum. Mol. Genet. 10, 1829-45) was used to allow transgene expression to be specifically switch off by removing doxycycline from the medium, without interfering with ongoing cellular protein synthesis. This is important because autophagy is protein-synthesis

dependent (Lawrence,B.P. and Brown,W.J. (1993) J. Cell Sci., 105, 473-480; Abeliovich,H. et al (2000) J. Cell Biol. 151, 1025-1034). The stable lines were induced for 8 hours (8h ON) and expression was then switched off by removing doxycycline from the medium for the next 24, 48 or 72 hours, which we have called 24, 48 or 72h OFF. The proportion of cells with aggregates peaked at 24h OFF and subsequently was reduced over time. The peak at 24h OFF may reflect a delay in the wash-out of doxycycline, or be a function of the kinetics of aggregation - aggregation occurs after a relatively long lag phase. Western blot analysis of these cells shows aggregates as a high molecular weight band in the stack at 48h OFF but these have largely disappeared at 72h OFF. The reduction in aggregates from 24 to 48 to 72 hours correlated with a reduction in the amounts of soluble protein. In order to test the effects of 3-MA or BafA1 on the turnover of aggregates, the cells were induced for 8 hours, then switched off for 48 or 72 hours with these treatments. Treatment with either of the inhibitors (72h OFF+3-MA and 72h OFF+BafA1) resulted in increased numbers of aggregate containing cells when compared to time-matched control (72h OFF). This is also supported by the results of the western blot -which showed that 3-MA or BafA1 resulted in increased aggregate formation, as seen by the high molecular weight fraction on the stack, which persists even 72 hours after switching off the expression (compared with time-matched untreated controls). These results provide indication that inhibiting the autophagy-lysosomal pathway interferes with the degradation of the mutant HD exon 1 protein/aggregates, which is consistent with the transient transfection experiments in COS-7 cells.

Induction of autophagy decreases aggregation and cell death in mammalian cells expressing Q74 and A19

The effect of inducing autophagy with an antifungal macrolide antibiotic, rapamycin, was examined. Treatment with rapamycin resulted in a decrease in the proportions of aggregate-containing cells and cell death in COS-7 cells expressing A19 but no significant change was observed in cells expressing Q74 after 48 hours of transfection (Fig. 3A). The aggregates formed by Q74 at 48h were much larger than at 24 hours and the aggregation rate in the untreated Q74 cells was higher when compared to the A19 aggregates (Fig. 3A). Thus, Q74 may aggregate more rapidly than A19 and more quickly form highly stable structures that are relatively resistant to rapamycin (autophagy). We therefore repeated the experiment at 24 hours post transfection with Q74, when fewer cells have formed aggregates (Fig. 3B). Under these conditions, rapamycin reduced the proportions of cells with aggregates and cell death (Fig. 3B). We performed similar experiments in the stable inducible PC12 cell line expressing EGFP-Q74. The cells were induced for 8 hours and then expression was switched off for 24, 48 or 72 hours, with or without rapamycin treatment. The rate at which protein clearance occurred was measured by the loss of fluorescence and also by comparing the protein levels on a western blot. Treatment with rapamycin resulted in a loss of fluorescence ((i) 72h OFF+Rap vs. 72h OFF) and also a decrease in protein levels on a western blot when compared to the untreated control. Since rapamycin is a slow inhibitor of protein synthesis, we checked whether inhibition of protein synthesis by treatment with cycloheximide had a similar effect on Q74 clearance and aggregation. In contrast to rapamycin, cycloheximide alone increased cell fluorescence and the number of

visible aggregates (72h OFF+Cycloheximide). Indeed, cycloheximide abrogated the effect of rapamycin on these cells (72h OFF+Rap&Cycloheximide), consistent with the observation that cycloheximide causes a drastic reduction in autophagy-induced protein degradation (Abeliovich,H. et al (2000) J. Cell Biol. 151, 1025-1034).

Effect of proteasome inhibition by epoxomicin

The effect of epoxomicin, a potent and selective proteasome inhibitor (Meng,L. et al (1999) Proc. Natl. Acad. Sci. U S A, 96, 10403-10408) was tested in COS-7 cells expressing Q74 or A19 and in PC12 cells stably expressing Q74. Immunofluorescence revealed a change in morphology of Q74 or A19 aggregates in COS-7 cells treated with epoxomicin. As described previously with Q74 and lactacystin (Wyttenbach, A. et al (2000) supra), there were more aggregates in each aggregate-containing cell. However, epoxomicin treatment did not affect the proportion of Q74 expressing cells with aggregates or apoptotic nuclear morphology. This is in contrast to lactacystin, which increases the proportion of Q74-expressing cells with aggregates. However, epoxomicin did increase the proportion of COS-7 cells expressing A19 with aggregates (Fig. 4). PC12 cells stably expressing Q74 were induced for 8 hours and expression was switched off for 48 hours without (48h OFF) or with treatment with epoxomicin (48h OFF+Epox) or lactacystin (48h OFF+Lac). Surprisingly, epoxomicin resulted in a reduction in the number of aggregate-containing cells when compared to control, even though it caused an obvious increase in cellular fluorescence. This was confirmed by western blot analysis of epoxomicin-treated cells which revealed a marked increase in the soluble fraction but failed to

detect any high molecular weight bands corresponding to aggregates. This is in contrast to lactacystin, which increased Q74 aggregation immunocytochemically (48h OFF+Lac) and both the soluble and insoluble fractions on western blots. A marked increase was observed in the heat shock protein 70 on epoxomicin treatment, consistent with inhibition of the proteasome (Kim,D., Kim,S.H. and Li,G.C. (1999) Biochem. Biophys. Res. Com., 254, 264-268).

Establishment of inducible α-synuclein cell lines.

Stable, inducible lines were made for human wildtype, A30P and A53T α-synuclein in PC12 (rat phaeochromocytoma) cells using the TetOn system, where addition of doxycycline switches on transgene expression. Two different clonal lines were selected for each form of α-synuclein, on the basis of low background transgene expression and high inducibility.

α-Synuclein localisation was examined in the induced cells. α-synuclein was found to be evenly distributed across the cells, often with a vesicular pattern of staining. The diffuse cytoplasmic and nuclear localisation of α-synuclein was similar to previous observation with this protein in PC12 cells (Rideout, H. J. et al (2001) *J. Neurochem.* **78**, 899908). At the light microscope level, it was unclear if the vesicular structures were aggregates. We did not see very large aggregates characteristic of polyglutamine and polyalanine expansions for either wildtype, A30P or A53T, even after expression of α-synuclein for 10 days. The staining for α-synuclein in uninduced cells was below the level of detection when analysed with confocal microscopy

using the same settings that gave clear signals for induced cells. However, α-synuclein immunoreactivity in the uninduced cells was observed when the gain was increased - this staining is likely to reflect predominantly endogenous α-synuclein in the cells, since there is minimal leakiness of transgene expression in uninduced cells.

Cell death was examined by FACS analysis and inspection of nuclear morphology after DAPI staining. No significant change in numbers of dead cells was observed after induction of α-synuclein expression in any of the lines expressing wildtype, A30P or A53T. In both cycling and differentiated cells, cell death was < 10 % at all times studied up to 10 days of induction (assessment by DAPI staining for wildtype, A30P or A53T).

α-Synuclein levels were determined by western blotting using an antiHA antibody to enable specific detection of the transgene products. All blots were performed at least twice with each clonal cell line. Expression of α-synuclein was switched on with doxycycline and then switch off expression by removing doxycycline from the medium, in order to follow α-synuclein degradation. Significantly lower levels of α-synuclein were observed 72 hours after expression was switched off, compared to the time point at which doxycycline was removed.

<u>α-Synuclein is degraded by the proteasome in our cell model</u>

α-Synuclein expression was switched on for 24 hours, then doxycycline was removed and the proteasome inhibitors epoxomicin or lactacystin added for 48 hours. We consistently saw an increase in α-synuclein levels with

treatment with the proteasome inhibitors epoxomicin or lactacystin, in both cycling and differentiated cells in all cell lines. Addition of these drugs did not cause increased cell death or overt aggregate formation.

α-Synuclein is degraded by autophagy

3Methyladenine (3MA) inhibits autophagy at the sequestration stage, where a double membrane structure forms around a portion of the cytosol. Bafilomycin A1 (baf A1) is a vacuolar ATPase inhibitor that interferes with the autophagosome-lysosome fusion step and rapamycin is an antifungal macrolide antibiotic that stimulates autophagy.

Cell lines were induced for 24 hours, then doxycycline was removed and the drugs were added for 48 hours. The trends we observed were similar in cells treated with drugs for 24 and 72 hours. Inhibiting autophagy with 3MA and baf A1 led to an obvious increase of α-synuclein levels in the A53T lines. These inhibitors only induced slight changes, at best, in α-synuclein levels in mitotic wildtype and A30P lines (e.g. 3MA with wildtype). In differentiated cells, the A53T lines again showed greater susceptibility to accumulating α-synuclein after treatment with these inhibitors. The densitometry values of α-synuclein bands (as a function of tubulin) were compared for 3MA and baf A1 treatments to those for control cells and calculated the fold increase in α-synuclein levels in the cells after drug treatment. Both inhibitors had significant effects on A30P accumulation (A30P + 3MA: 1.13 fold increase (+/-0.04 SE); p=0.04 (n=4 experiments; paired t tests in all cases); A30P + baf A1: 1.19 fold increase (+/-0.015 SE); p=0.006 (n=3)). While the trend for wildtype α-synuclein suggests that these

inhibitors were impairing its degradation, the data did not reach significance (wt + 3MA: 1.48 fold increase (+/-0.36 SE); p=0.26 (n=5); wt + baf A1: 1.12 fold increase (+/-0.08 SE); p=0.23 (n=4)).

α-Synuclein is seen in vesicles with autophagic morphology

The subcellular localisation of α-synuclein in the cell lines was examined using immunogold electron microscopy. An antibody to the Hatag in the α-synuclein transgene products was used to detect α-synuclein. Gold α-synuclein labelling was apparent over vesicles with autophagic morphology i.e. the vesicles had morphologies consistent with those described in previous studies of autophagy (Mizushima, N., et al . (2001) *J. Cell Biol.* **152,** 657667), Huntington's disease (Kegel, K. B. et al (2000) *J. Neurosci.* **20,** 72687278) and α-synuclein (Stefanis, L. (2001) *J. Neurosci.* **21** 95499560). (In these studies, vesicles were identified as autophagic on similar morphologic grounds, in the absence of specific antibodies).

α-synuclein was observed either "free" or associated with electron dense bodies of around 100nm in diameter. There were often 2 or more gold particles over these bodies, which could indicate α-synuclein microaggregates. Such bodies were also seen in the cytoplasm, sometimes associated with α-synuclein.

These electron dense bodies may be dense core granules transporting neurotransmitters. Confocal immunofluorescence studies showed that α-synuclein (wildtype, A30P and A53T) was localised inside acidic vacuoles labelled using LysoTracker Red. LysoTracker Red

labels acid compartments including degradative autophagic vacuoles and lysosomes. α-synuclein was not excluded from these compartments and speckles of α-synuclein immunoreactivity are clearly visible within these vacuoles.

In the results described herein, polyQ and polyA expansions have been used as models for aggregate-prone proteins caused by codon reiteration mutations. Our results indicate that autophagy is indeed involved in the degradation of our model proteins, as these accumulated when cells were treated with different inhibitors acting at distinct stages of the autophagy-lysosome pathway, in two different cell lines. These inhibitors are used to test the role of autophagy in different contexts. Furthermore, rapamycin, which stimulates autophagy, enhanced the clearance of our aggregate-prone proteins. Rapamycin also reduced the appearance of aggregates and the cell death associated with the polyQ and polyA expansions.

The results herein also show that α-synuclein is degraded by both the proteasome and autophagy in inducible PC12 cell lines. Western blotting data showed that autophagy is a clearance route for this protein and this was confirmed by by observations of wildtype, A53T and A30P species in autophagic vesicles by EM. This is the first report that autophagy as well as the proteasome is a route for α-synuclein degradation. Furthermore, the autophagy inducer rapamycin is shown to increase clearance of all forms of α-synuclein.

Rapamycin is already used clinically (e.g. for the treatment of transplant rejection) (Chan,L. et al (2001)

Am. J. Kidney Dis., 38, S2-S9), and the data shown herein indicates that this drug and related analogues are candidates for therapeutic investigation in diseases characterised by protein aggregation, including codon iteration mutation disorders such as polyQ and polyA expansion disorders, including Huntington's disease, and also α-synucleinopathies such as Parkinson's disease.

Claims:

1. A method of treating a protein conformational disorder in an individual comprising:
stimulating autophagy activity in the individual.

2. A method according to claim 1 wherein the protein conformational disorder is characterised by cytoplasmic protein aggregation.

3. A method according to claim 1 or claim 2 comprising administering an autophagy inducing agent to said individual.

4. A method according to claim 3 wherein the autophagy inducing agent is a rapamycin macrolide.

5. A method according to claim 4 wherein the rapamycin macrolide is rapamycin.

6. A method according to claim 4 wherein the rapamycin macrolide is a rapamycin analogue.

7. A method according to claim 6 wherein the rapamycin analogue is selected from the group consisting of 40-O-(2-hydroxy)ethyl-rapamycin, 32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-40-O-(2-hydroxyethyl)-rapamycin, 16-O-pent-2-ynyl-32-(S)-dihydro-rapamycin and 16-O-pent-2-ynyl-32-(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin

8. A method according to any one of the preceding claims wherein the disorder is a codon reiteration mutation disorder.

9. A method according to claim 8 wherein the disorder is a polyQ expansion disorder.

10. A method according to claim 9 wherein the polyQ expansion disorder is selected from the group consisting of Huntington's disease, spinocerebellar ataxias types 1, 2, 3, 6, 7, and 17, Kennedy's disease and dentatorubral-pallidoluysian atrophy.

11. A method according to claim 8 wherein the disorder is a polyA expansion disorder.

12. A method according any one of claims 1 to 7 wherein the disorder is an α-synucleiopathy.

13. A method according to claim 12 wherein the disorder is selected from the group consisting of Parkinson's disease, LB variant Alheimer's disease, LB dementia.

14. A method according to any one of claims 1 to 7 wherein the disorder is a prion disorder.

15. A method according to claim 14 wherein the prion disorder is CJD.

16. Use of an autophagy inducing agent in the manufacture of a medicament for use in the treatment of a protein conformational disorder in an individual.

17. Use according to claim 16 wherein the protein conformational disorder is characterised by cytoplasmic protein aggregation.

18. Use according to claim 16 or claim 17 wherein the autophagy inducing agent is a rapamycin macrolide.

19. Use according to claim 18 wherein the rapamycin macrolide is rapamycin.

20. Use according to claim 18 wherein the autophagy inducing agent is a rapamycin analogue.

21. Use according to claim 20 wherein the analogue is selected from the group consisting of 40-O-(2-hydroxy)ethyl-rapamycin, 32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-rapamycin, 16-O-pent-2-ynyl-32-deoxo-40-O-(2-hydroxyethyl)-rapamycin, 16-O-pent-2-ynyl-32-(S)-dihydro-rapamycin and 16-O-pent-2-ynyl-32-(S)-dihydro-40-O-(2-hydroxyethyl)-rapamycin

22. Use according to any one of claims 16 to 21 wherein the disorder is a codon reiteration mutation disorder.

23. Use according to claim 22 wherein the disorder is a polyQ expansion disorder.

24. Use according to claim 23 wherein the polyQ expansion disorder is selected from the group consisting of Huntington's disease, spinocerebellar ataxias types 1, 2, 3, 6, 7, and 17, Kennedy's disease and dentatorubral-pallidoluysian atrophy.

25. Use according to claim 22 wherein the disorder is a polyA expansion disorder.

26. Use according to any one of claims 16 to 21 wherein the disorder is an α-synucleiopathy.

27. Use according to claim 26 wherein the disorder is selected from the group consisting of Parkinson's disease, LB variant Alheimer's disease, LB dementia.

28. Use according any one of claims 16 to 21 wherein the disorder is a prion disorder.

29. Use according to claim 28 wherein the prion disorder is CJD.

30. A method of identifying an agent useful in the treatment of a protein conformational disorder comprising;

contacting a mammalian cell with a test compound; and,

determining the autophagy activity of said cell, an increase in autophagy activity in the presence of said compound being indicative that the compound is a candidate agent for use in the treatment of a protein conformational disorder.

31. A method of producing an agent for the treatment of a protein conformational disorder comprising; modifying rapamycin to produce a rapamycin derivative; and; determining the autophagy inducing activity of said derivative.

32. A method according to claim 31 comprising determining the ability of said derivative to enhance the clearance of cytoplasmic protein aggregates.

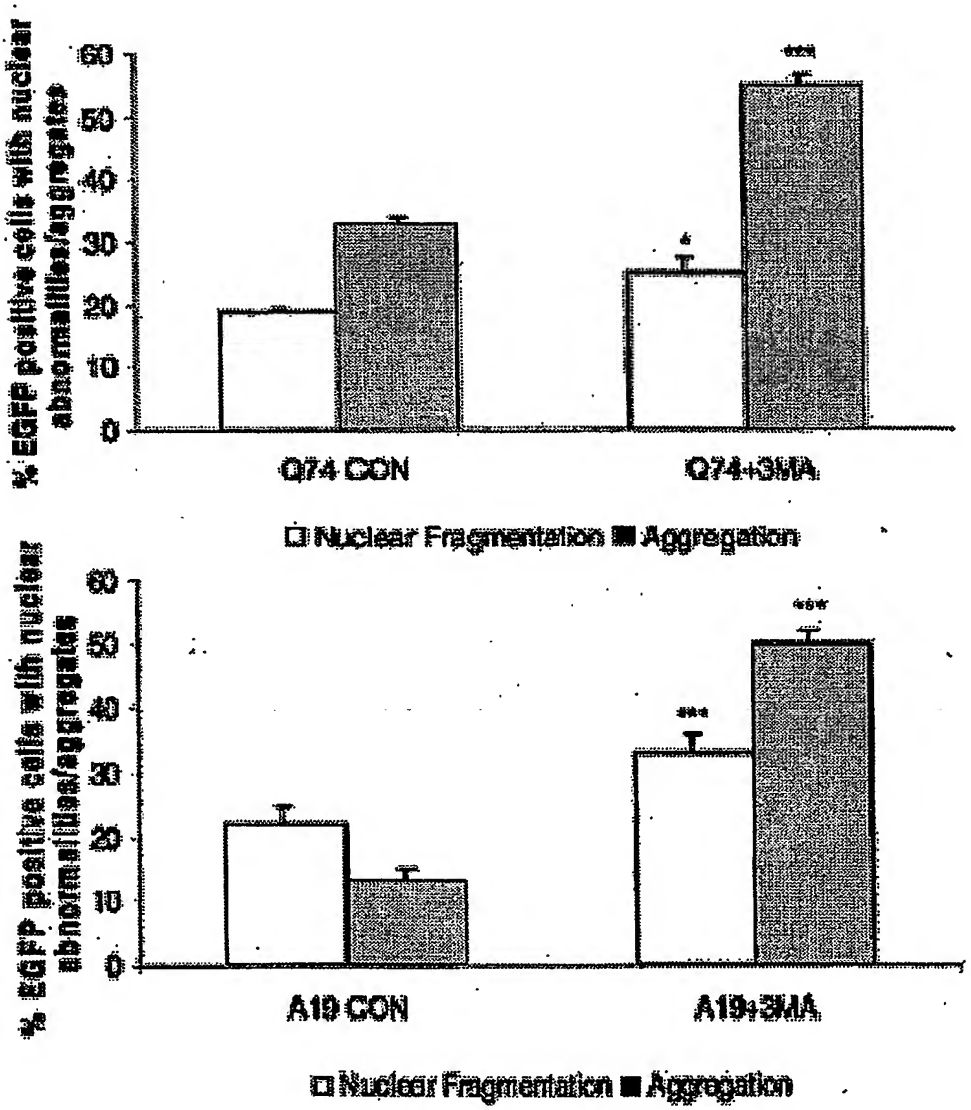

% EGFP positive cells with nuclear abnormalities/aggregates
0
10
20
30
40
50
60
Q74 CON
Q74+3MA
Nuclear Fragmentation
Aggregation
% EGFP positive cells with nuclear abnormalities/aggregates
0
10
20
30
40
50
60
A19 CON
A19+3MA
Nuclear Fragmentation
Aggregation

Figure 1

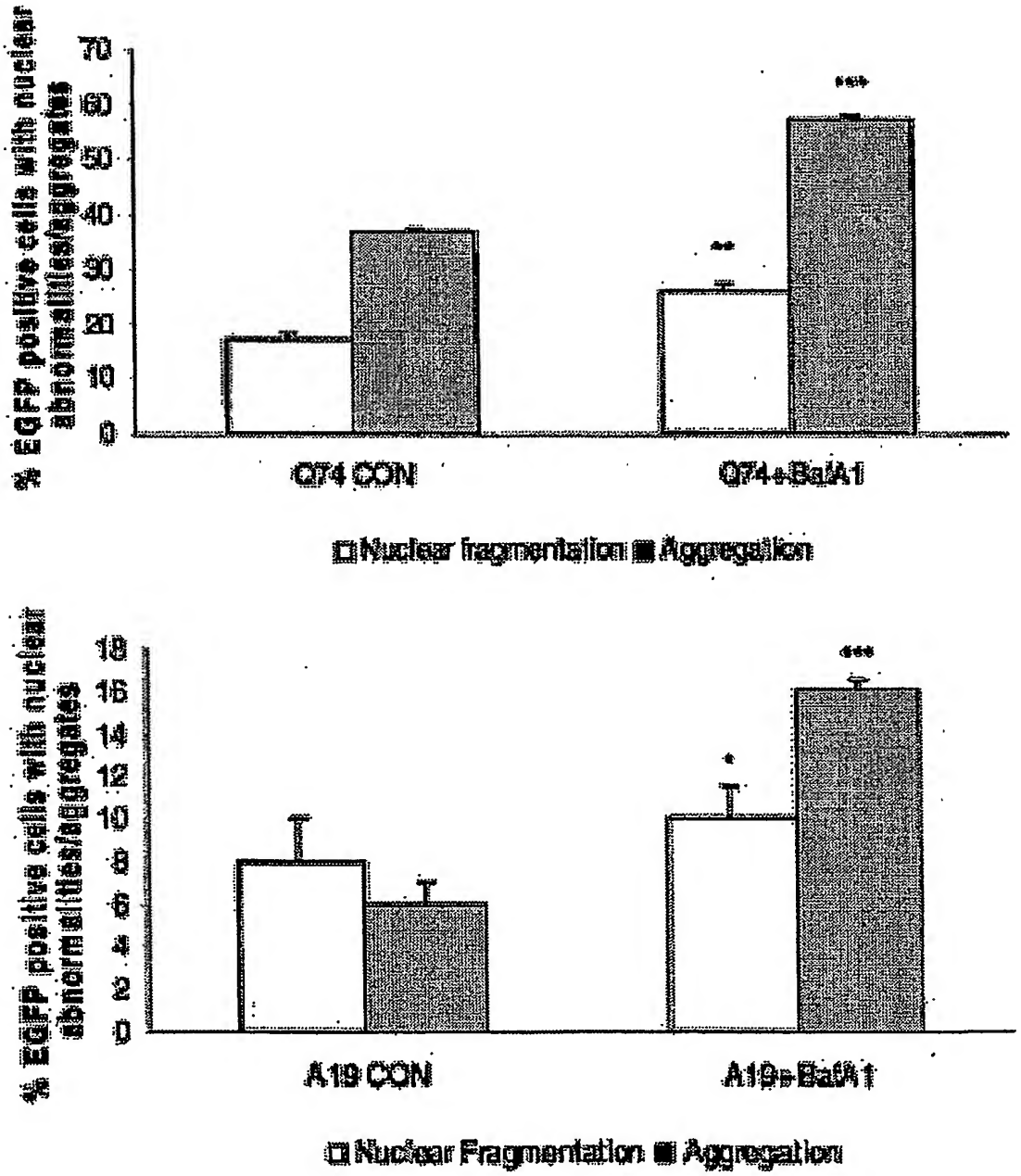

% EGFP positive cells with nuclear abnormalities/aggregates
0
10
20
30
40
50
60
70
Q74 CON
Q74+BclA1
Nuclear fragmentation
Aggregation
% EGFP positive cells with nuclear abnormalities/aggregates
0
2
4
6
8
10
12
14
16
18
A19 CON
A19+BclA1
Nuclear Fragmentation
Aggregation

Figure 2

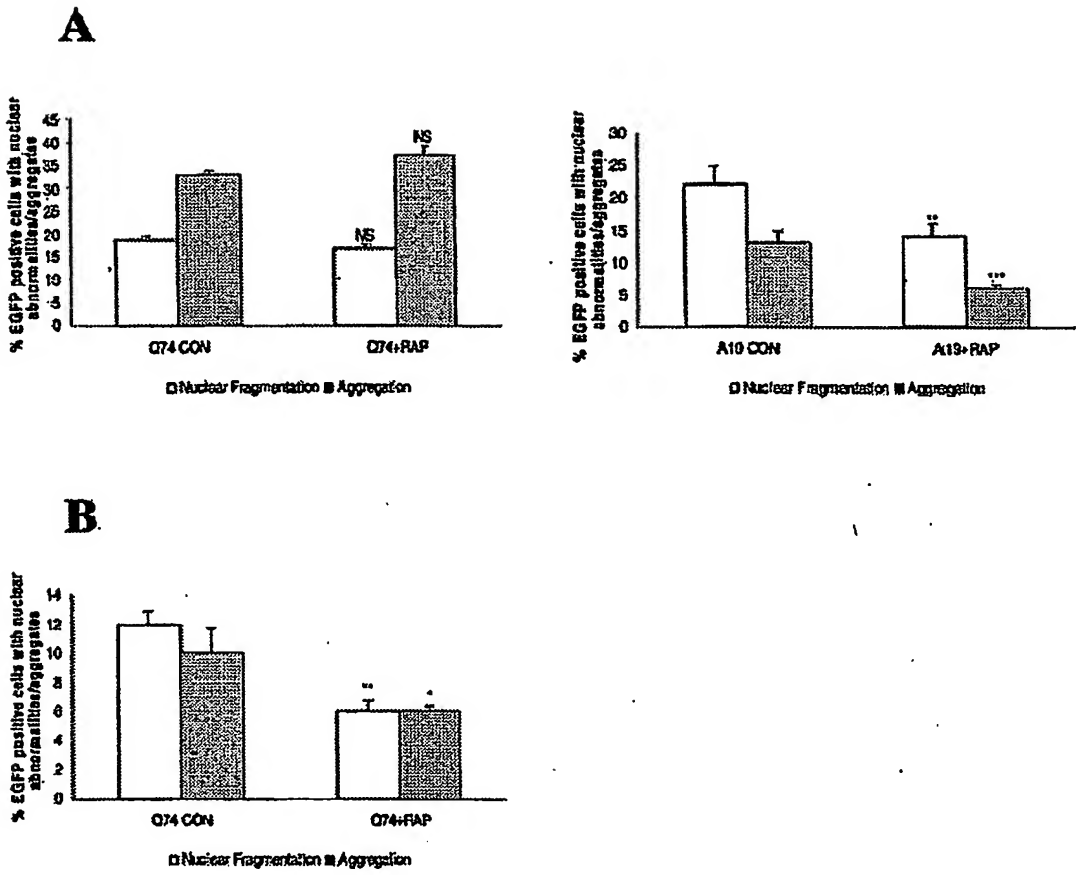

A
% EGFP positive cells with nuclear abnormalities/aggregates
NS
NS
Q74 CON
Q74+RAP
Nuclear Fragmentation
Aggregation
% EGFP positive cells with nuclear abnormalities/aggregates
A10 CON
A13+RAP
Nuclear Fragmentation
Aggregation
B
% EGFP positive cells with nuclear abnormalities/aggregates
Q74 CON
Q74+RAP
Nuclear Fragmentation
Aggregation

Figure 3

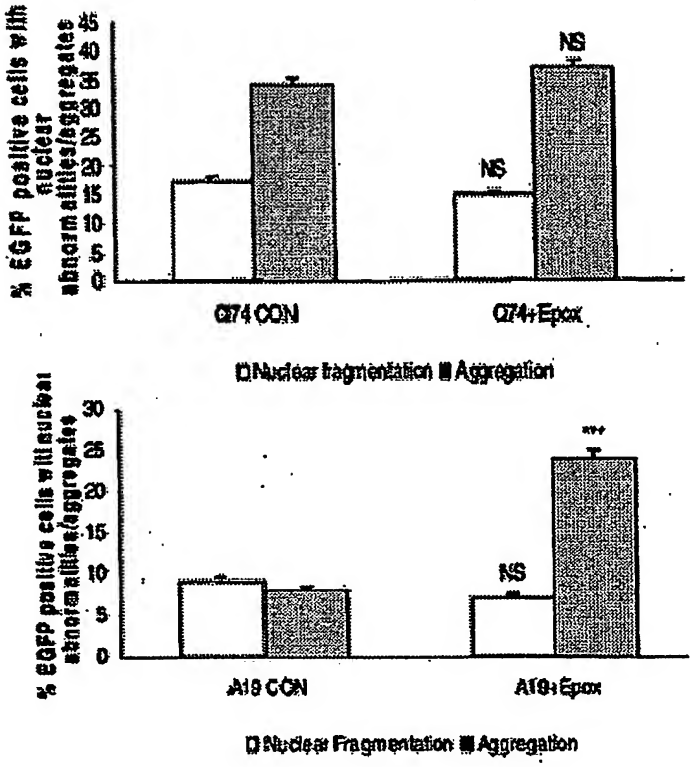

% EGFP positive cells with nuclear abnormalities/aggregates
0
5
10
15
20
25
30
35
40
45
NS
NS
G74 CON
G74+Epox
Nuclear fragmentation
Aggregation
% EGFP positive cells with nuclear abnormalities/aggregates
0
5
10
15
20
25
30
NS
A19 CON
A19+Epox
Nuclear Fragmentation
Aggregation

Figure 4

Abstract

Methods and Means for Treating Protein Conformational Disorders

This invention relates to the recognition that autophagy plays a key role in the clearance of the intracellular protein aggregates which characterise Protein Conformational Disorders, such as Huntington's disease and Parkinson's disease. Methods and uses of autophagy inducing agents, such as rapamycin macrolides, in the treatment of Protein Conformational Disorders, are described herein.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☑ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.